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Discovery of novel 5-(ethyl or hydroxymethyl) analogs of 2′-'up' fluoro (or hydroxyl) pyrimidine nucleosides as a new class of *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Mycobacterium avium* inhibitors

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ABSTRACT

Discovery of novel antimycobacterial compounds that work on distinctive targets and by diverse mechanisms of action is urgently required for the treatment of mycobacterial infections due to the emerging global health threat of tuberculosis. We have identified a new class of 5-ethyl or hydroxy (or methoxy) methyl-substituted pyrimidine nucleosides as potent inhibitors of *Mycobacterium bovis*, *Mycobacterium tuberculosis* (H37Ra, H37Rv) and *Mycobacterium avium*. A series of 2′-'up' fluoro (or hydroxy) nucleosides (**1, 2,4–6, 9, 10, 13, 16, 18, 21, 24**) was synthesized and evaluated for antimycobacterial activity. Among 2′-fluorinated compounds, 1-(3-bromo-2,3-dideoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethyluracil (**13**) exhibited promising activity against *M. bovis* and *Mtb* alone, and showed synergism when combined with isoniazid. The most active compound emerging from these studies, 1-(β-D-arabinofuranosyl)-4-thio-5-hydroxymethyluracil (**21**) inhibited *Mtb* (H37Ra) (MIC_{50} = 0.5 μg/mL) and *M. bovis* (MIC_{50} = 0.5 μg/mL) at low concentrations, and was ten times more potent against *Mtb* (H37Ra) than cycloserine (MIC_{50} = 5.0 μg/mL), a second line drug. It also showed an additive effect when combined with isoniazid. Compound **21** retained sensitivity against a rifampicin-resistant (H37Rv) strain of *Mtb* (MIC_{50} = 1 μg/mL) at concentrations similar to that for a rifampicin-sensitive (H37Rv) strain, suggesting that it has no cross-resistance to a first-line anti-TB drug. In addition, the replication of *M. avium* was also inhibited by **21** (MIC_{50} = 10 μg/mL). No cellular toxicity of **13** or **21** was observed up to the highest concentration tested (CC_{50} > 100 μg/mL). These observations offer promise for a new drug treatment regimen to augment and complement the current chemotherapy of TB.

1. Introduction

Tuberculosis (TB) is caused predominantly by *Mycobacterium tuberculosis* (*Mtb*), an obligate aerobic bacillus. The vast majority of TB infections are caused by *Mtb*, but other closely related mycobacteria, *Mycobacterium bovis* and *Mycobacterium avium* can also cause severe disease.[1–3] *M. bovis*, in attenuated form, has been used as BCG vaccine.[1] However, recently *M. bovis* infections have re-emerged and are causing TB in humans, particularly those who are immunosuppressed.[2,3] Further, multidrug resistant (MDR) strains of *M. bovis* have also been found that are resistant to a large number of anti-TB drugs.[2,3] Clinical management of *M. avium* infection is very difficult since many of the first-line anti-TB drugs are ineffective against them.[4–6]

TB-causing mycobacteria have evolved a highly efficient means of aerosol transmission, and its extent is reflected in the estimate that one third of the global population is latently infected with *Mtb*.[7] Around one in ten individuals in this infected population develop active TB. In 2009 an estimated 9.4 million new cases of TB were reported and 1.7 million people died.[8] The expanding threats of TB drug resistance and concurrent human infection with immunodeficiency virus (HIV) and TB, compound the already crippling burden of TB. HIV infection facilitates reactivation of *Mtb* and results in more rapid progression from infection to active disease. Thus, the HIV/AIDS epidemic has been the primary cause of the surge in TB cases over the past few decades.[9]

An outbreak of recently recognized 'extensively drug-resistant TB' (XDR-TB) threatens the control of TB globally by making it almost incurable.[9] Although, most of the XDR-TB cases have been reported among HIV-infected patients, there have been reports of XDR-TB among HIV-negative patients.[10] The currently available TB treatment is inadequate. The long duration of a complex

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treatment regimen and associated side effects result in treatment non-compliance. Insufficient treatment with the current drug regimen allows rapid emergence of resistance and continuous spread of the drug-sensitive as well as drug-resistant mycobacterial strains.[11] Clearly, there is an urgent need to discover new treatments for TB by either modifying the application of existing agents or introducing new drugs. New anti-TB drugs are required to permit shorter treatment times, reduce toxic side effects, and treat MDR and XDR-TB.

The identification of novel targets for developing new anti-TB drugs has been facilitated by the complete genome sequencing of *Mtb*.[12] Several enzymes involved in purine and pyrimidine metabolism, and DNA and RNA synthesis differ significantly between humans and *Mtb*.[13] Therefore, nucleoside and nucleotide analogs may interfere these targets thereby disrupting nucleic acid biosynthetic pathways of mycobacteria.

An important area of anti-TB drug design is the investigation of a new class of pyrimidine nucleosides that possess substitutions at the C-5 position of the pyrimidine base including novel glycosyl moieties which exhibit potent and selective antimycobacterial activity. In our recent studies[14], we reported various 5-methyl pyrimidine nucleosides substituted at 2′ and/or 3′ positions which exhibited significant antimycobacterial activity against *Mtb* (H37Ra and H37Rv) (MIC_{50} = 1–25 μg/mL range). In this article, we report the antimycobacterial activities of a novel class of 5-ethyl and 5-hydroxymethyl derivatives of related pyrimidine nucleosides and their potential as potent and selective anti-TB agents.

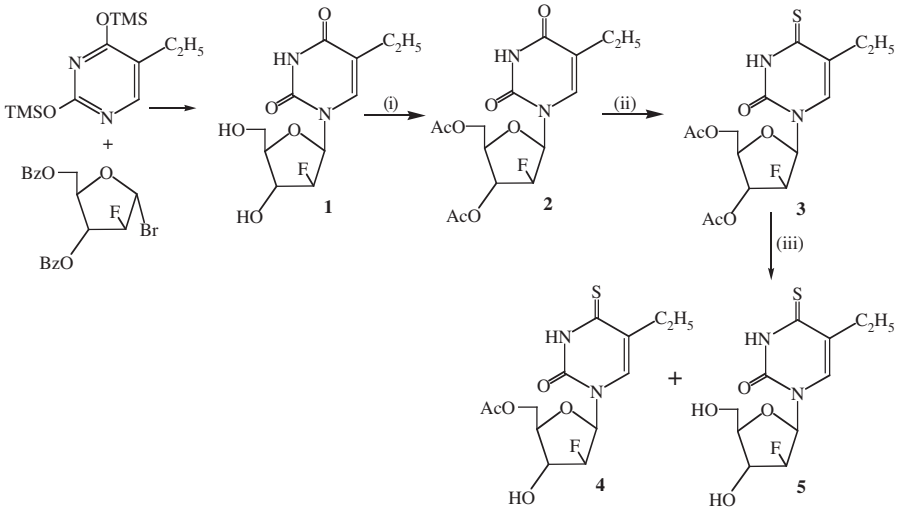
5-Ethyl-2′-deoxyuridine (EDU), a 'natural' thymidine analog with the same base-pairing property as thymidine,[15] has been reported to be non-mutagenic in several organisms.[16] In the carbohydrate portion of EDU, replacement of hydrogen with fluorine (which is strongly electronegative and has the same van der Waals radius as H) has been used to obtain favorable biological properties. It was subsequently shown that the introduction of a 2′-fluoroarabino substituent enhanced the chemotherapeutic potential of EDU.[17] The 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl) derivative of EDU was found to be highly active against several herpes viruses with much lower toxicity towards host cells compared to other fluorinated nucleosides, partly due to its non-inhibitory effect on cellular DNA synthesis. Importantly, this modification lead to immediate and sustained inhibition of hepatitis B virus replication in a woodchuck model with no apparent toxic effects.[17,18]

5-Hydroxymethyl-2′-deoxyuridine has been reported as a potent inhibitor of vaccinia virus and herpes simplex virus.[19] Meldrum et al[20] reported that it had selective cytotoxic activity on a variety of tumor cell lines. In contrast, 5-hydroxymethyl analogs of AZT (3′-azido-2′,3′-dideoxythymidine) and FLT (3′-fluoro-2′,3′-dideoxythymidine), had lower anti-HIV activity than AZT and FLT. However, 5-hydroxymethyl-3′-azido-2′,3′-dideoxyuridine and 5-hydroxymethyl-3′-fluoro-2′,3′-dideoxyuridine were significantly less toxic than AZT and FLT and no toxicity to host cells was found up to a concentration of 400 μM[21] In addition, the 5-hydroxymethyl analogs of thymidine inhibited the replication of *Escherichia coli* (*E. coli*)[22], and Bacimethrin, a hydroxymethyl analog of thymine is a known antibacterial agent for *Salmonella typhimurium* (*S. typhimurium*) and *Salmonella enterica* (*S. enterica*). Bacimethrin has been shown to inhibit the bacterial thiamine biosynthetic pathway.[23]

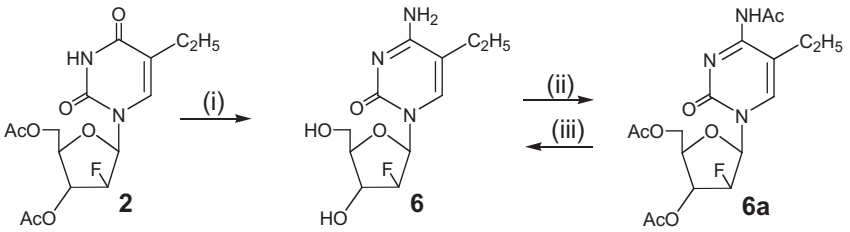
Herein we report the synthesis and antimycobacterial activity of a new class of 5-ethyl and 5-(hydroxymethyl or methoxymethyl) substituted pyrimidine analogs with various 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl) (**1**, **2**, **4–6**), 2,3′-anhydro derivative (**9**), 1-(2-deoxy-2-fluoro-3′-(un)substituted-β-D-arabinofuranosyl) (**10**, **13**, **16**) and 1-β-D-arabinofuranosyl (**18**, **21**, **24**) moieties against *M. bovis*, *Mtb* and *M. avium*, in vitro. Among these compounds, 1-(3-bromo-2,3-dideoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethyluracil (**13**) provided promising in vitro activity against *M. bovis* and *Mtb* alone and in combination with isoniazid. 1-(β-D-arabinofuranosyl)-4-thio-5-hydroxymethyluracil (**21**) displayed the most potent inhibition of *Mtb* H37Ra (MIC_{50} = 0.5 μg/mL), H37Rv (MIC_{50} = 1.0 μg/mL) and *M. bovis* (MIC_{50} = 0.5 μg/mL). This compound also retained activity for a rifampicin-resistant *Mtb* strain H37Rv at a similar concentration (MIC_{50} = 1.0 μg/mL). In addition, it was encouraging to note that compound **21** exhibited activity against *M. avium* (MIC_{50} = 10.0 μg/mL).

2. Chemistry

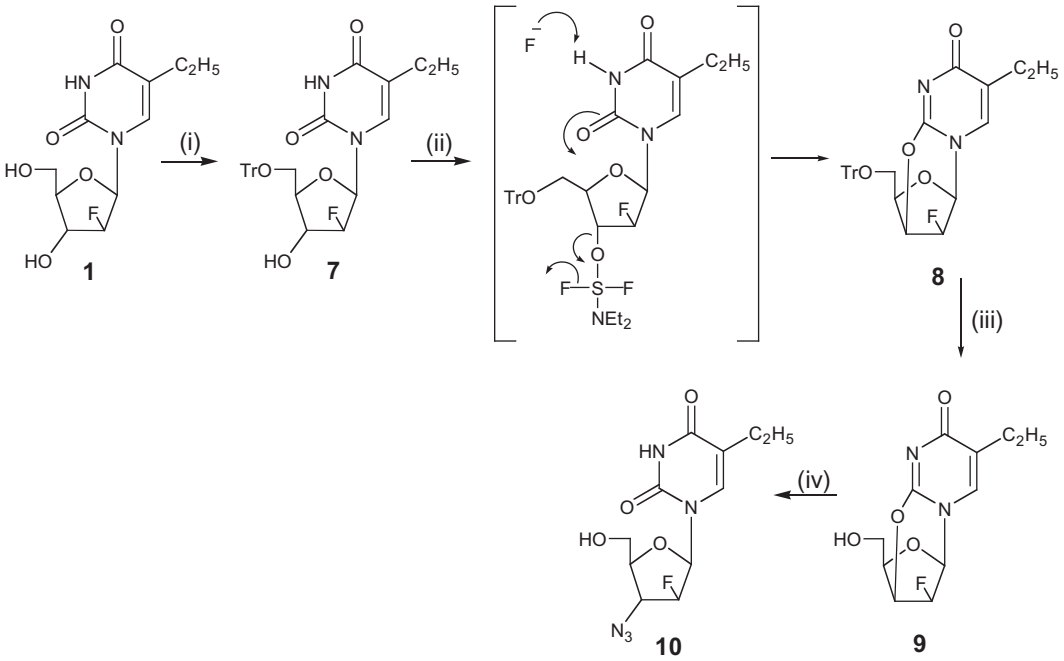
2′-Fluoroarabinosyl nucleoside, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethyluracil (**1**) was prepared by a coupling reaction of silylated 5-ethyluracil and the appropriate furanosyl bromide.[24] Acetylation of **1** with acetic anhydride in anhydrous pyridine at room temperature yielded 1-(3,5-di-O-acetyl-2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethyluracil (**2**) in 99% yield. The compound **2** on thiation with Lawesson's reagent in anhydrous 1,4-dioxane under refluxing conditions gave the crude 4-thio 3′-5′-di-acetylated product **3** which upon deacetylation with methanolic



Scheme 1. Reagents and conditions: (i) Acetic anhydride, anhydrous pyridine, 0 °C to room temperature, 24 h, 99% yield; (ii) Lawesson's reagent, anhydrous 1,4-dioxane, reflux, 3 h; (iii) methanolic ammonia, room temperature, 4 h (**4**, 25%; **5**, 43% yield).



Scheme 2. Reagents and conditions: (i) (a) 2,4,6-Triisopropylbenzenesulfonyl chloride, 4-(dimethylamino)pyridine, anhydrous Et_3N, anhydrous CH_3CN, room temperature, 60 h, (b) NH_4OH, room temperature, 24 h; (ii) acetic anhydride, anhydrous pyridine, 0 °C to room temperature, 24 h; (iii) K_2CO_3, MeOH, 0 °C, 30 min, 32% yield.



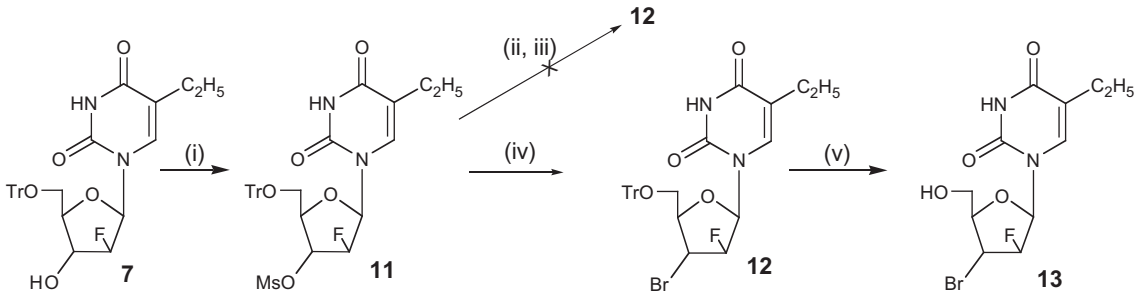
Scheme 3. Reagents and conditions: (i) TrCl, 4-(dimethylamino)pyridine, anhydrous pyridine, 80 °C, 8 h, 89% yield; (ii) DAST, anhydrous CH_2Cl_2, -12 °C to room temperature, 6 h, 78% yield; (iii) 80% AcOH, 90 °C, 30 min, 82% yield; (iv) NaN_3, anhydrous DMF, 125–130 °C, 12 h, 16% yield.

ammonia at room temperature for 4 h provided 1-(5-*O*-acetyl-2-deoxy-2-fluoro-β-D-arabinofuranosyl)-4-thio-5-ethyluracil (**4**) and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-4-thio-5-ethyluracil (**5**) in 25% and 43% yields, respectively (Scheme 1). The removal rate of acetyl group at 5′-O-position is expected to be faster than acetyl group at 3′-O-position. The formation of **4** could have been resulted due to an intramolecular migration of acetyl moiety from 3′-O-position to 5′-O-position. 1-(2-Deoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethylcytosine (**6**) was prepared by the reaction of **2** with 2,4,6-triisopropylbenzenesulphonyl chloride in the presence of 4-(dimethylamino)pyridine (DMAP) and Et_3N followed by treatment with NH_4OH at room temperature. The obtained compound **6** was impure and it was difficult to remove the impurities by repeated column chromatography. Therefore, it was converted to 3′,5′-N-triacetylated derivative **6a** which, after purification, was deblocked with K_2CO_3 in methanol at 0 °C. The desired pure product **6** was obtained in an overall yield of 32%, starting from **2** (Scheme 2).

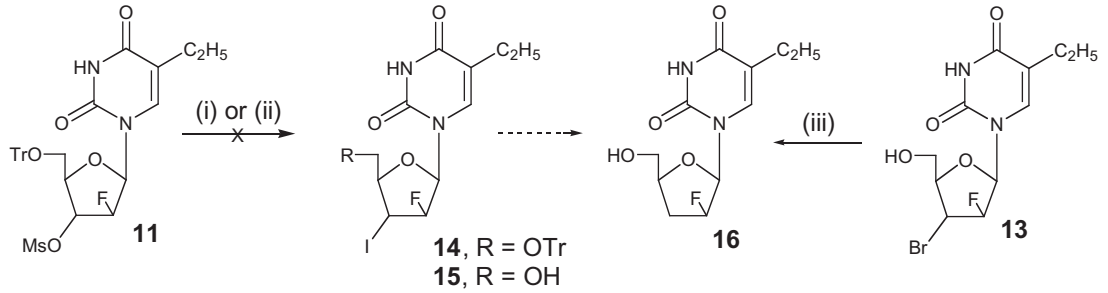
Reaction of **1** with triphenylmethyl chloride in dry pyridine at 80 °C in the presence of DMAP followed by treatment of the obtained 5′-*O*-trityl derivative (**7**) with (diethylamino)sulfur trifluoride (DAST) in anhydrous CH_2Cl_2 gave 2,3′-anhydro nucleoside **8** in 78% yield. Compound **8**, upon reaction with 80% AcOH at 90 °C, yielded the deblocked 2,3′-anhydro product **9** in 82% yield. Azidation of **9** with NaN_3 in dry DMF at 125–130 °C provided **10** in 16% yield (Scheme 3).

1-(3-Bromo-2,3-dideoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethyluracil (**13**) was synthesized from compound **7** (Scheme 4). Compound **7** upon treatment with methanesulphonyl chloride (mesyl chloride) in anhydrous pyridine at 0 °C afforded the 3′-*O*-mesyl-5′-*O*-trityl derivative **11** in 97% yield. Reaction of **11** with LiBr in anhydrous CH_3CN under reflux or in anhydrous DMF at 110 °C, despite prolonged heating (48 h), did not provide the desired 3′-bromo product **12**. However, our attempt using NaBr as a source of bromide anion (Br^-) was successful. Thus, compound **11** upon reaction with NaBr in anhydrous DMF at 110 °C for 24 h gave the 3′-bromo derivative **12** in 34% yield which after detritylation with 80% AcOH at 90 °C yielded the target nucleoside **13** in 60% yield.

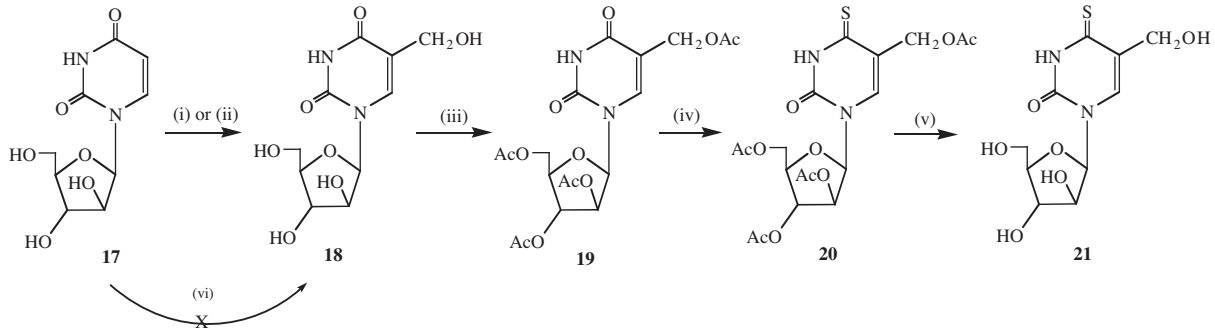
The 3′-deoxy derivative, 1-(2,3-dideoxy-2-fluoro-β-D-*threo*-pentofuranosyl)-5-ethyluracil (**16**) was synthesized as illustrated in Scheme 5. Initially we planned to synthesize **16** via 3′-iodo derivatives (**14** and **15**), since 3′-iodo derivatives have been reported to be easily deiodinated to give the 3′-deoxy compound.[25] However, our attempts to synthesize the 3′-iodo derivative **14** failed under various reaction conditions (NaI/anhydrous DME, reflux, 24 h or NaI/anhydrous DMF, 110 °C, 24 h). Subsequently, we attempted synthesis of the 3′-deoxy derivative **16** by the reductive debromination of compound **13**. Compound **13** upon treatment with Et_3N, in the



Scheme 4. Reagents and conditions: (i) Mesyl chloride, anhydrous pyridine, 0–5 °C, 48 h, 97%, yield; (ii) LiBr, anhydrous CH_3CN, reflux, 48 h; (iii) LiBr, anhydrous DMF, 110 °C, 48 h; (iv) NaBr, anhydrous DMF, 110 °C, 24 h, 34% yield; (v) 80% AcOH, 90 °C, 30 min, 60% yield.



Scheme 5. Reagents and conditions: (i) NaI, anhydrous DME, reflux, 24 h; (ii) NaI, anhydrous DMF, 110 °C, 24 h; (iii) H_2, 10% Pd-C, Et_3N, MeOH, room temperature, 2 h, 45% yield.



Scheme 6. Reagents and conditions: (i) Paraformaldehyde, 0.5 N KOH, 65 °C, 52 h, 9% yield; (ii) 37% formaldehyde, 0.5 N KOH, 65 °C, 18 h, 30% yield; (iii) acetic anhydride, anhydrous pyridine, 0 °C to room temperature, 42% yield; (iv) Lawesson's reagent, anhydrous 1,4-dioxane, reflux, 3.5 h, 40% yield; (v) K_2CO_3, MeOH, 0 °C, 1% yield; (vi) paraformaldehyde, 0.5 N HCl, reflux, 12 h.

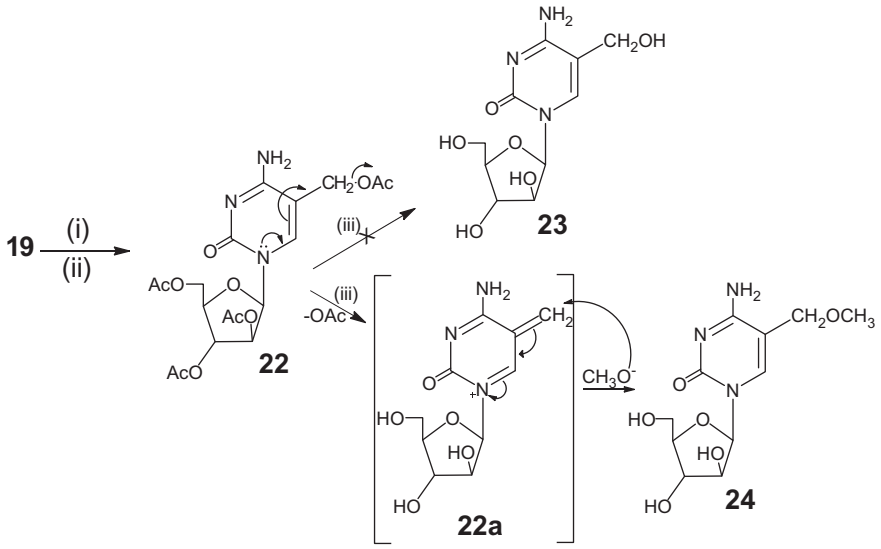
presence of 10% Pd-C, in methanol under hydrogen atmosphere gave the desired compound **16** in 45% yield.

1-β-D-Arabinofuranosyluracil (**17**) was heated at 65 °C with paraformaldehyde in 0.5 N aqueous KOH for 52 h to obtain the 5-hydroxymethyl derivative **18**, but this method provided **18** in poor yield (9%). In order to optimize the yield of **18**, we reacted compound **17** with 37% formaldehyde in base as well as with paraformaldehyde using acidic (0.5 N HCl) conditions. It was found that the yield of **18** was improved to 30% when 37% formaldehyde in basic conditions was used, whereas the starting material (**17**) was decomposed when it was heated with paraformaldehyde under acidic conditions. In order to synthesize the 4-thio analog **21**, compound **18** was acetylated using acetic anhydride in anhydrous pyridine to give a tetra-*O*-acetyl derivative **19** which was then reacted with Lawesson's reagent in dry 1,4-dioxane to yield the protected 4-thio derivative **20** in 40% yield. Compound **20** was deprotected using K_2CO_3 in dry methanol to afford the target compound **21**. (Scheme 6)

Reaction of **19** with *p*-toluenesulfonyl chloride, Et_3N and 1-methylpiperidine in dry acetonitrile at 0 °C followed by treatment with NH_4OH at 0 °C provided **22**, which upon treatment with K_2CO_3 in methanol at 0 °C, did not yield the desired product **23** but resulted in 1-(β-D-arabinofuranosyl)-5-methoxymethylcytosine (**24**) in 54% yield. The possible mechanism for the conversion of **22** to **24** may include the formation of α,β-unsaturated imine intermediate **22a** under basic conditions followed by Michael type addition of methanol at C-5 of **22a** to give **24** (Scheme 7).

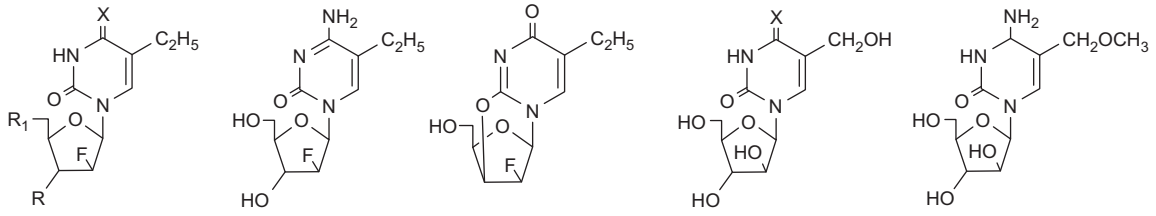
3. Results and discussion

The antimycobacterial activities for this new class of 5-ethyl (**1, 2, 4–6, 9, 10, 13, 16**), 5-hydroxymethyl (**18, 21**) and 5-methoxymethyl (**24**) pyrimidine analogs were determined against mycobacteria (*M. bovis*, *Mtb* H37Ra, *M. avium*) using the microplate alamar blue assay (MABA)[26] at 0.5–100 μg/mL concentrations. The results are summarized in Table 1. Cycloserine, rifampicin,



Scheme 7. Reagents and conditions: (i) *p*-Toluenesulfonyl chloride, Et_3N, 1-methylpiperidine, anhydrous CH_3CN, 0 °C, 4 h; (ii) NH_4OH, 0 °C, 15 min, 30% yield; (iii) K_2CO_3, dry MeOH, 0 °C, 30 min, 54% yield.

Table 1
In vitro antimycobacterial activity of test compounds against *M. bovis, Mtb* and *M. avium*



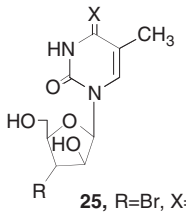
Compd	*X*	R	R_1	Antimycobacterial activity % inhibition[a] (concentration μg/mL)		
				M. bovis (BCG)	*Mtb* (H37Ra)	*M. avium* (ATCC 25291)
1	O	OH	OH	0	0	0
2	O	$OCOCH_3$	$OCOCH_3$	75 (100), 55 (50)	68 (100), 55 (50)	35 (100)
4	S	OH	$OCOCH_3$	80 (100), 65 (50), 50 (10)	73 (100), 60 (50), 50 (10)	50 (100), 25 (50)
5	S	OH	OH	65 (100), 55 (50)	60 (100), 50 (50)	35 (100), 25 (50)
6	—	—	—	50 (100)	50 (100)	0
9	—	—	—	25 (100, 50)	25 (100, 50)	50 (100), 25 (50)
10	O	N_3	OH	0	0	0
13	O	Br	OH	80 (100), 70 (50), 60 (10), 50 (5)	78 (100), 65 (50), 56 (10), 50 (5)	0
16	O	H	OH	25 (100)	25 (100)	25 (100)
18	O	—	—	0	0	0
21	S	—	—	100 (100, 50), 90 (10), 75 (5), 50 (0.5)	100 (100, 50), 90 (10), 70 (5), 50 (0.5)	73 (100), 58 (50), 50 (10)
24	—	—	—	80 (100), 60 (50), 50 (10)	80 (100), 60 (50), 50 (10)	20 (100)
Cycloserine	—	—	—	ND[b]	100 (50), 80 (15–20), 60 (10), 50 (5)	ND
Rifampicin	—	—	—	100 (0.5)	100 (0.5)	90 (2)
Clarithromycin	—	—	—	ND	ND	100 (2)

[a] Antimycobacterial activity was determined at concentrations 100, 50, 25, 10, 5, 2, 1 and 0.5 μg/mL.
[b] ND = not determined.

and clarithromycin were used as reference drugs. The compounds that exhibited promising inhibition against the wild-type strain of *M. bovis* were further evaluated against wild-type and rifampicin-resistant strains of *Mtb* (H37Rv).

Among the 5-ethyl series of compounds described here 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-4-thio-5-ethyluracil (**5**, MIC_{50} = 50 μg/mL) and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethylcytosine (**6**, MIC_{50} = 100 μg/mL) exhibited moderate inhibition of *Mtb* (H37Ra), in contrast to 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethyluracil (**1**). However, it is interesting to note that the acetate derivatives (**2, 4**) of **1** and **5** showed increased anti-TB activity when compared to **1** and **5**. This enhanced activity could partly be attributed to their increased lipophilicity. From this series of compounds, the 3′-bromo analog, 1-(3-bromo-2,3-dideoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethyluracil (**13**) displayed the best antimycobacterial activity against both *M. bovis* and *Mtb* (MIC_{50} = 5 μg/mL) ,equal to the second-line anti-TB drug cycloserine (MIC_{50} = 5 μg/mL, Table 1). This observation was consistent with our previous studies where an analogous compound, 1-(3-bromo-3-deoxy-β-D-arabinofuranosyl)thymine (**25**), also exhibited the most potent activity against

Mtb (MIC_{50} = 1 μg/mL)[14] and *M. bovis* (40% inhibition at 1 μg/mL, unpublished results). Although the activity of **13** was not improved over earlier compound **25** as expected, it was encouraging to note that the efficacy of **13** was not completely lost but was only 5-fold less than compound **25** against both *M. bovis* and *Mtb*. This suggests that substitution of a methyl group for an ethyl group on the uracil ring, and replacement of the hydroxyl at the 2′-position by fluorine are still acceptable by both mycobacterial species. The other 5-ethyl derivatives, *O*-2,3′-anhydro-1-(2-deoxy-2-fluoro-β-D-lyxofuranosyl)-5-ethyluracil (**9**), 1-(3-azido-2,3-dideoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethyluracil (**10**) and 1-(2,3-dideoxy-2-fluoro-β-D-threo-pentofuranosyl)-5-ethyluracil (**16**) had almost no, or very low, inhibitory action against any mycobacteria, suggesting that 3′-bromo substituent plays an important role in antimycobacterial activity against *Mtb* in pyrimidine nucleosides. These results are in agreement with our previous observations made with thymidine analogs.[14]



25, R=Br, X=O
26, R=OH, X=O,
27, R=OH, X=S

Of the 5-hydroxymethyl derivatives, 4-thio analog, 1-(β-D-arabinofuranosyl)-4-thio-5-hydroxymethyluracil (**21**), and 4-amino analog, 1-(β-D-arabinofuranosyl)-5-methoxymethylcytosine (**24**), showed potent anti-TB activity whereas 4-oxo analog, 1-(β-D-arabinofuranosyl)-5-hydroxymethyluracil (**18**) was found to be inactive. Similar correlations were also observed in the 5-ethyl series of compounds **1**, **5** and **6**, although **21** and **24** possessed much higher activity. Encouragingly, anti-TB activity displayed by **21** (MIC_{50} = 0.5 μg/mL) was superior to **13** (MIC_{50} = 5 μg/mL), the most active agent of 5-ethyl series. Further, the MIC_{50} exhibited by compound **21** was ten times lower than the reference drug cycloserine (MIC_{50} = 5 μg/mL). In addition, compound **21** also showed marked inhibition of *M. avium* (MIC_{50} = 10 μg/mL). These findings were surprising since in our earlier studies[27] 1-(β-D-arabinofuranosyl)thymine (**26**) and 1-(β-D-arabinofuranosyl)-4-thio-thymine (**27**) were found to be inactive, suggesting that the hydroxymethyl group at the C-5 position has an influence on antimycobacterial activity. However, inactivity of compound **18** indicates that 5-hydroxymethyl group together with a thio substituent at the C-4 position contribute to the potent antimycobacterial activity against *Mtb* as well as *M. avium*.

The most active inhibitor, **21**, was also evaluated for its activity towards drug-susceptible and rifampicin-resistant strains of *Mtb*, H37Rv. Intriguingly, compound **21** demonstrated an MIC_{50} of 1.0 μg/mL against both drug-susceptible and drug-resistant mycobacteria. In these assays, rifampicin showed no activity against the drug-resistant strain at 2.0 μg/mL whereas isoniazid provided 100% inhibition at 1.0 μg/mL.

We wanted to understand potential and possible interactions of our new class of nucleoside analogs with current antimycobacterial drugs, so we selected compounds **13** and **21** to test in combination with isoniazid to determine their effect on the growth of *Mtb* (H37Ra) in vitro. Compounds **13** and **21** were used at 10 and 5 μg/mL, respectively, and isoniazid at 0.5, 0.1 and 0.05 μg/mL. It was encouraging to note that **13** provided synergistic inhibition of *Mtb* (70% inhibition at 10 μg/mL) whereas **21** showed an additive effect (82% inhibition at 5 μg/mL) when combined with isoniazid at 0.1 μg/mL. Isoniazid showed only 10% inhibition at 0.1 μg/mL by itself whereas **13** and **21** exhibited 56% and 90%, and 50% and 70% inhibition at 10 and 5 μg/mL, respectively. Synergy was defined as $x/y < 1/z$, where x is the growth index of the combination of drugs, y is the lowest growth index of a single drug of the combination and z is the number of drugs in combination. For a two drug combination, a quotient <0.5 indicates synergistic action whereas a quotient of 0.5–1 reflects an additive effect.[28] The synergy quotient for the combination of compound **13** and isoniazid is 0.33, which is lower than 0.5, suggesting a synergistic interaction. For the combination of **21** and isoniazid, the synergy quotient was 0.8, reflecting an additive effect. This study suggests that our novel nucleosides are acting at a different target, not interacting antagonistically, and would be effective when combined with current drugs.

The XTT and ^{3}H-thymidine incorporation assays were performed to evaluate the toxicity of investigated compounds (**1, 2, 4–6, 9, 10, 13, 16, 18, 21, 24**) in vitro against a human hepatoma cell line (Huh-7). These compounds were not toxic up to the highest concentration tested, 100 μg/mL (CC_{50} > 100 μg/mL). These results were significant as the modifications sought at the C-5 position of the base and at 2′ and/or 3′ positions of the sugar moiety of pyrimidine nucleoside derivatives described in this manuscript did not contribute to enhanced toxicity as compared to compounds reported in our previous studies.[14,27]

Due to the re-emergence of TB infection globally, and the resistance of mycobacteria to existing drugs, there is an urgent need to identify novel antimycobacterial compounds that work by new mechanisms of action. In this work, a new class of pyrimidine nucleosides was identified which has promising anti-TB activity against several mycobacterial species [Bacillus Calmette Guerin (BCG), *Mtb* H37Ra, H37Rv, *M. avium*] and has no cytotoxicity. Compounds **13** and **21** emerged as the most efficacious analogs. Compound **13** was found to be as active as cycloserine, a second-line TB drug and demonstrated a synergistic effect in combination with a first-line drug, isoniazid. Compound **21** has activity against various mycobacterial species and with different isolates of *Mtb*. It exhibited activity towards both drug-susceptible and drug-resistant strains and was 10 times more potent than the reference drug cycloserine. Thus the new inhibitors identified in this report hold promise to be used alone or in combination for the development of a new class of agents for TB infections.

Our results with 5-ethyl- and 5-hydroxymethyl series of pyrimidine nucleosides indicate that not only C-5, but other modifications e.g., substituents on the carbohydrate moiety, and C-4 position of the base, also play an important role in the determination of antimycobacterial activity. These observations provide us with an impetus for designing a new series in the near future which would integrate features of compounds **13** and **21**, and relevant compounds from our previous work.

4. Experimental section

Melting points were determined with an electrothermal melting point apparatus and are uncorrected. ^{1}H NMR spectra were determined for samples in Me_2SO-d_6, $CDCl_3$ or CD_3OD on a Bruker AM 300 spectrometer using TMS as an internal standard. Chemical shifts are given in ppm relative to TMS and signals are described as s (singlet), d (doublet), t (triplet), br (broad signal), q (quartet), m (multiplet), dm (doublet of multiplet), and dd (doublet of doublets). The assignment of all exchangeable protons (OH, NH) was confirmed by the addition of D_2O. All of the final compounds had >95% purity except **6a** (>95% as a water adduct), determined by microanalysis. Microanalysis results were within ±0.4% of the theoretical values for all elements listed unless otherwise indicated. Silica gel column chromatography was carried out using Merck 7734 silica gel (100–200 μM particle size). Thin–layer chromatography

(TLC) was performed with Machery-Nagel Alugram SiL G/UV silica gel slides (20 μM thickness). 1-β-D-arabinofuranosyluracil (**17**) was purchased from Aldrich.

4.1. 1-(3,5-Di-*O*-acetyl-2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethyluracil (2)

To an ice-cooled solution of **1** (1.0 g, 3.65 mmol) in anhydrous pyridine (25 mL) was added acetic anhydride (0.82 mL, 8.72 mmol) and the reaction mixture was stirred at 0 °C for 1 h. The stirring was continued at room temperature for 23 h. Solvent was removed in vacuo and the crude product thus obtained was purified on a silica gel column using MeOH/$CHCl_3$ (2:98, v/v) as the eluent to give **2** (1.30 g, 99%) as a solid; mp 50–52 °C; $[\alpha]_D$ +41.08 (*c* 0.33, MeOH); ^{1}H NMR ($CDCl_3$): δ 1.15 (t, *J* = 7.37 Hz, 3H, CH_3), 2.13 (s, 3H, CH_3), 2.17 (s, 3H, CH_3), 2.38 (q, *J* = 7.34 Hz, 2H, CH_2), 4.23–4.52 (m, 3H, H-4′, H-5′), 5.10 (dd, $J_{2',F}$ = 49.9 Hz, 2.74 Hz, 1H, H-2′), 5.24 (dd, $J_{3',F}$ = 16.64 Hz, 2.70 Hz, 1H, H-3′), 6.22 (dd, $J_{1',F}$ = 22.44 Hz, 2.82 Hz, 1H, H-1′), 7.29 (m, 1H, H-6), 8.53 (br s, 1H, NH). ES-MS (+ve mode) = 359.1 $(M+H)^+$; Anal. Calcd for $C_{15}H_{19}FN_2O_7$: C 50.28, H 5.34, N 7.82. Found: C 50.48, H 5.47, N 7.65.

4.2. 1-(5-*O*-Acetyl-2-deoxy-2-fluoro-β-D-arabinofuranosyl)-4-thio-5-ethyluracil (4) and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-4-thio-5-ethyluracil (5)

To a dried mixture of **2** (2.60 g, 7.26 mmol) and Lawesson's reagent (4.40 g, 10.88 mmol) was added anhydrous 1,4-dioxane (50 mL). The reaction mixture was refluxed for 3 h, cooled to rt and concentrated in vacuo to give the crude reaction mixture. This mixture was then stirred with methanolic ammonia (40 mL) at room temperature for 4 h. The solvent was removed in vacuo and the crude product thus obtained was purified on a silica gel column using MeOH/$CHCl_3$ (2:98, v/v) as the eluent to give **4** (0.61 g, 25%) as a solid; mp 108–110 °C; ^{1}H NMR (DMSO-d_6): δ 1.06 (t, *J* = 7.32 Hz, 3H, CH_3), 2.06 (s, 3H, CH_3), 2.48 (q, *J* = 7.32 Hz, 2H, CH_2), 4.03–4.36 (m, 4H, H-3′, H-4′, H-5′), 5.08 (dt, $J_{2',F}$ = 52.19 Hz, 3.66 Hz, 1H, H-2′), 6.08 (d, *J* = 4.58 Hz, 1H, 3′-OH), 6.13 (dd, $J_{1',F}$ = 17.39 Hz, 3.97 Hz, 1H, H-1′), 7.41 (s, 1H, H-6), 12.88 (s, 1H, NH); ES-MS (+ve mode) = 333.1 $(M+H)^+$; ES-MS (−ve mode) = 331.1 $(M-H)^-$; Anal. Calcd for $C_{13}H_{17}FN_2O_5S$: C 46.98, H 5.16, N 8.43. Found: C 46.77, H 5.28, N 8.31.

Further elution with MeOH/$CHCl_3$ (3:97, v/v) yielded **5** (0.91 g, 43%) as a solid; mp 95–97 °C; $[\alpha]_D$ +150.39 (*c* 0.33, MeOH); ^{1}H NMR (DMSO-d_6): δ 1.06 (t, *J* = 7.33 Hz, 3H, CH_3), 2.48 (q, *J* = 7.33 Hz, 2H, CH_2), 3.64 (m, 2H, H-5′), 3.81 (m, 1H, H-4′), 4.26 (dm, $J_{3',F}$ = 20.14 Hz, 1H, H-3′), 5.11 (dt, $J_{2',F}$ = 52.79 Hz, 4.27 Hz, 1H, H-2′), 5.22 (br s, 1H, 5′-OH), 5.93 (d, *J* = 4.88 Hz, 1H, 3′-OH), 6.13 (dd, $J_{1',F}$ = 12.82 Hz, 4.88 Hz, 1H, H-1′), 7.72 (s, 1H, H-6), 12.83 (s, 1H, NH); ES-MS (+ve mode) = 291.1 $(M+H)^+$; ES-MS (−ve mode) = 289.1 $(M-H)^-$; Anal. Calcd for $C_{11}H_{15}FN_2O_4S$: C 45.51, H 5.21, N 9.65. Found: C 45.65, H 5.32, N 9.47.

4.3. 1-(2-Deoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethylcytosine (6)

To a dried mixture of **2** (1.30 g, 3.63 mmol), 2,4,6-triisopropylbenzenesulfonyl chloride (3.47 g, 11.46 mmol) and 4-(dimethylamino)pyridine (0.70 g, 5.73 mmol) was added anhydrous acetonitrile (50 mL) followed by anhydrous Et_3N (2.66 mL, 19.07 mmol). The reaction mixture was stirred at room temperature for 60 h. NH_4OH (40 mL) was added and the stirring was continued at room temperature for a further 24 h. The solvent was removed in vacuo and the crude product thus obtained was purified on a silica gel column using MeOH/$CHCl_3$ (15:85, v/v) as the eluent to give **6** as an impure syrup (1.38 g). The syrup was dried, acetylated with acetic anhydride (0.71 mL, 7.54 mmol) in anhydrous pyridine (30 mL) at room temperature. The triacetylated derivative was purified on a silica gel column using MeOH/$CHCl_3$ (3:97, v/v) as the eluent to give **6a** (0.6 g) as a syrup.

To an ice cooled solution of **6a** (0.6 g, 1.50 mmol) in methanol (30 mL) was added K_2CO_3 (1.04 g, 7.52 mmol) and the reaction mixture was stirred at 0 °C for 30 min. The solvent was removed in vacuo and the crude product thus obtained was purified on a silica gel column using MeOH/$CHCl_3$ (20:80, v/v) as the eluent to give **6** (0.32 g, 32%) as a solid; mp 140–142 °C; $[\alpha]_D$ +107.76 (*c* 0.28, MeOH); ^{1}H NMR (DMSO-d_6): δ 1.02 (t, *J* = 7.35 Hz, 3H, CH_3), 2.24 (q, *J* = 7.36 Hz, 2H, CH_2), 3.53–3.65 (m, 2H, H-5′), 3.76 (m, 1H, H-4′), 4.18 (dm, $J_{3',F}$ = 19.41 Hz, 1H, H-3′), 4.95 (dm, $J_{2',F}$ = 52.63 Hz, 1H, H-2′), 5.12 (br s, 1H, 5′-OH), 5.85 (br s, 1H, 3′-OH), 6.11 (dd, $J_{1',F}$ = 17.27 Hz, 4.08 Hz, 1H, H-1′), 6.92 (br s, 1H, NH), 7.35 (br s, 1H, NH), 7.43 (s, 1H, H-6). Anal. Calcd for $C_{11}H_{16}FN_3O_4$ +0.85 H_2O: C 45.78, H 6.18. Found: C 46.08, H 5.96.

4.4. 1-(2-Deoxy-2-fluoro-5-*O*-trityl-β-D-arabinofuranosyl)-5-ethyluracil (7)

To a dried mixture of **1** (3.0 g, 10.94 mmol), trityl chloride (4.58 g, 16.43 mmol) and 4-(dimethylamino)pyridine (0.20 g, 1.64 mmol) was added in anhydrous pyridine (70 mL) and the reaction mixture was heated at 80 °C for 8 h. The solvent was removed in vacuo and the crude product thus obtained was purified on a silica gel column using MeOH/$CHCl_3$ (5:95, v/v) as the eluent to give **7** (5.0 g, 89%) as a solid; mp 212–214 °C; ^{1}H NMR (DMSO-d_6): δ 0.83 (t, *J* = 7.32 Hz, 3H, CH_3), 2.03 (q, *J* = 7.32 Hz, 2H, CH_2), 3.17–3.35 (m, 2H, H-5′), 3.97 (m, 1H, H-4′), 4.24–4.39 (m, 1H, H-3′), 5.04 (dm, $J_{2',F}$ = 52.49 Hz, 1H, H-2′), 5.95 (d, *J* = 4.88 Hz, 1H, 3′-OH), 6.17 (dd, $J_{1',F}$ = 17.70 Hz, 4.27 Hz, 1H, H-1′), 7.25–7.45 (m, 16H, 5′-*O*-trityl and H-6), 11.48 (s, 1H, NH); ES-MS (+ve mode) = 539.2 $(M+Na)^+$; ES-MS (−ve mode) = 515.2 $(M-H)^-$.

4.5. 2,3′-*O*-Anhydro-1-(5-*O*-trityl-2-deoxy-2-fluoro-β-D-lyxofuranosyl)-5-ethyluracil (8)

To a solution of **7** (0.66 g, 1.28 mmol) in anhydrous dichloromethane (30 mL) at −12 °C was added DAST (1.35 mL, 10.24 mmol) and the reaction mixture was stirred at −12 °C for 30 min, followed by stirring at room temperature for 5.5 h. The reaction mixture was poured into 5% $NaHCO_3$ (50 mL) and the aqueous layer was extracted with EtOAc (3 × 25 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated to give the crude product, which was purified on a silica gel column using EtOAc as the eluent to give **8** (0.50 g, 78%) as a syrup; ^{1}H NMR (DMSO-d_6): δ 1.08 (t, *J* = 7.32 Hz, 3H, CH_3), 2.23 (q, *J* = 7.32 Hz, 2H, CH_2), 3.13 (d, *J* = 6.71 Hz, 2H, H-5′), 4.62 (m, 1H, H-4′), 5.43 (t, *J* = 3.05 Hz, 1H, H-3′), 5.92 (dm, $J_{2',F}$ = 51.26 Hz, 1H, H-2′), 6.02 (m, 1H, H-1′), 7.23–7.41 (m, 15H, 5′-*O*-trityl), 7.63 (s, 1H, H-6); ES-MS (+ve mode) = 499.2 $(M+H)^+$.

4.6. *O*-2,3′-Anhydro-1-(2-deoxy-2-fluoro-β-D-lyxofuranosyl)-5-ethyluracil (9)

A solution of **8** (0.50 g, 1 mmol) in 80% aqueous AcOH (40 mL) was heated at 90 °C for 30 min. The solvent was removed in vacuo and the crude product thus obtained was purified on a silica gel column using MeOH/$CHCl_3$ (15:85, v/v) as the eluent to give **9** (0.21 g, 82%) as a solid; mp 202–204 °C (dec.); $[\alpha]_D$ −40.64 (*c* 0.31, MeOH); ^{1}H NMR (DMSO-d_6): δ 1.04 (t, *J* = 7.32 Hz, 3H, CH_3), 2.23 (q, *J* = 7.32 Hz, 2H, CH_2), 3.44–3.58 (m, 2H, H-5′), 4.36 (m, 1H, H-4′), 5.16 (t, *J* = 5.49 Hz, 1H, 5′-OH), 5.31 (t, *J* = 3.05 Hz, 1H, H-3′), 5.89 (dt, $J_{2',F}$ = 50.66 Hz, 3.66 Hz, 1H, H-2′), 5.95 (m, 1H, H-1′), 7.57 (s, 1H, H-6); ES-MS (+ve mode) = 257.1 $(M+H)^+$; Anal. Calcd for

$C_{11}H_{13}FN_2O_4$: C 51.56, H 5.11, N 10.93. Found: C 51.41, H 5.18, N 10.87.

4.7. 1-(3-Azido-2,3-dideoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethyluracil (10)

To a dried mixture of **9** (0.10 g, 0.39 mmol) and NaN_3 (0.026 g, 0.4 mmol) was added anhydrous DMF (10 mL). The reaction mixture was heated at 125–130 °C for 12 h. Solvent was removed in vacuo and the crude product thus obtained was purified on a silica gel column using EtOAc/Hexane (50:50, v/v) as the eluent to give **10** (0.019 g, 16%) as syrup; ^{1}H NMR (DMSO-d_6): δ 1.03 (t, J = 7.32 Hz, 3H, CH_3), 2.23 (q, J = 7.32 Hz, 2H, CH_2), 3.69 (m, 2H, H-5′), 3.83 (m, 1H, H-4′), 4.52 (ddd, $J_{3',F}$ = 21.97 Hz, 7.93 Hz, 5.49 Hz, 1H, H-3′), 5.40 (dt, $J_{2',F}$ = 53.10 Hz, 5.49 Hz, 1H, H-2′), 5.42 (br s, 1H, 5′-OH), 6.17 (dd, $J_{1',F}$ = 9.76 Hz, 5.49 Hz, 1H, H-1′), 7.59 (s, 1H, H-6), 11.45 (br s, 1H, NH); Anal. Calcd for $C_{11}H_{14}FN_5O_4$: C 44.15, H 4.72, N 23.40. Found: C 44.41, H 4.77, N 23.16.

4.8. 1-(2-Deoxy-2-fluoro-3-*O*-mesyl-5-*O*-trityl-β-D-arabinofuranosyl)-5-ethyluracil (11)

To an ice cooled solution of **7** (5.65 g, 10.94 mmol) in anhydrous pyridine (60 mL) was added mesyl chloride (1.69 mL, 21.82 mmol) dropwise with stirring. The reaction mixture was kept in the refrigerator for 48 h. After the addition of water (2 mL), the solvent was evaporated and the resulting residue was dissolved in $CHCl_3$ (100 mL), washed with water (2 × 30 mL) and dried over anhydrous Na_2SO_4. The solvent was removed in vacuo and the residue thus obtained was purified on a silica gel column using MeOH/$CHCl_3$ (3:97, v/v) as the eluent to give **11** (6.30 g, 97%) as a solid; mp 90–92 °C; ^{1}H NMR (DMSO-d_6): δ 0.86 (t, J = 7.32 Hz, 3H, CH_3), 2.04 (q, J = 7.32 Hz, 2H, CH_2), 3.24 (s, 3H, CH_3), 3.30–3.47 (m, 2H, H-5′), 4.25 (m, 1H, H-4′), 5.41 (m, 1H, H-3′), 5.55 (dm, $J_{2',F}$ = 56.15 Hz, 1H, H-2′), 6.26 (dd, $J_{1',F}$ = 16.48 Hz, 4.27 Hz, 1H, H-1′), 7.25–7.45 (m, 16H, 5′-*O*-trityl and H-6), 11.55 (s, 1H, NH); ES-MS (+ve mode) = 617.2 $(M+Na)^+$; ES-MS (−ve mode) = 593.2 $(M-H)^-$.

4.9. 1-(3-Bromo-2,3-dideoxy-2-fluoro-5-*O*-trityl-β-D-arabinofuranosyl)-5-ethyluracil (12)

To a dried mixture of **11** (0.60 g, 1.0 mmol) and NaBr (1.99 g, 19.34 mmol) was added anhydrous DMF (30 mL). The reaction mixture was heated at 110 °C for 24 h. The solvent was removed in vacuo and the crude product thus obtained was purified on a silica gel column using EtOAc/Hexane (30:70, v/v) as the eluent to give **12** (0.20 g, 34%) as a syrup; ^{1}H NMR ($CDCl_3$): δ 0.98 (t, J = 7.32 Hz, 3H, CH_3), 2.22 (q, J = 7.32 Hz, 2H, CH_2), 3.47 (m, 2H, H-5′), 4.33 (m, 1H, H-4′), 4.50 (ddd, $J_{3',F}$ = 23.19 Hz, 5.49 Hz, 1.83 Hz, 1H, H-3′), 5.31 (dm, $J_{2',F}$ = 53.10 Hz, 1H, H-2′), 6.36 (dd, $J_{1',F}$ = 18.31 Hz, 3.66 Hz, 1H, H-1′), 7.28–7.50 (m, 16H, 5′-*O*-trityl and H-6), 8.65 (br s, 1H, NH); ES-MS (+ve mode) = 601.1 (M+Na)+; ES-MS (−ve mode) = 577.1 $(M-H)^-$.

4.10. 1-(3-Bromo-2,3-dideoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethyluracil (13)

Detritylation of **12** using the procedure as described for **9** provided **13**. Yield 60%. Compound **13** was recrystallized from EtOH. mp 62–64 °C; $[\alpha]_D$ +50.15 (*c* 0.31, MeOH); ^{1}H NMR (DMSO-d_6): δ 1.02 (t, J = 7.32 Hz, 3H, CH_3), 2.22 (q, J = 7.32 Hz, 2H, CH_2), 3.72 (m, 2H, H-5′), 4.17 (m, 1H, H-4′), 4.55 (ddd, $J_{3',F}$ = 20.75 Hz, 7.93 Hz, 6.10 Hz, 1H, H-3′), 5.44 (t, J = 5.49 Hz, 1H, 5′-OH), 5.61 (dt, $J_{2',F}$ = 53.10 Hz, 6.10 Hz, 1H, H-2′), 6.29 (dd, $J_{1',F}$ = 8.54 Hz, 6.10 Hz, 1H, H-1′), 7.63 (s, 1H, H-6), 11.48 (s, 1H, NH). Anal. Calcd for $C_{11}H_{14}BrFN_2O_4$: C 40.02, H 4.76, N 7.78. Found: C 39.82, H 4.45, N 8.07.

4.11. 1-(2,3-Dideoxy-2-fluoro-β-D-*threo*-pentofuranosyl)-5-ethyl uracil (16)

To a solution of **13** (0.05 g, 0.15 mmol) in MeOH (10 mL) was added Et_3N (0.1 mL) and 10% Pd-C (0.035 g) and the reaction mixture was stirred at room temperature under H_2 atmosphere for 2 h. The reaction mixture was filtered through celite and the filtrate obtained was concentrated in vacuo. The crude product was purified on preparative TLC using MeOH/$CHCl_3$ (5:95, v/v) as the eluent to give **16** (0.017 g, 45%) as a solid; mp 120–122 °C; $[\alpha]_D$ +75.34 (*c* 0.28, MeOH); ^{1}H NMR (DMSO-d_6): δ 1.02 (t, J = 7.32 Hz, 3H, CH_3), 2.01–2.20 (m, 2H, H-3′), 2.23 (q, J = 7.32 Hz, 2H, CH_2), 3.51–3.68 (m, 2H, H-5′), 4.09 (m, 1H, H-4′), 5.07 (t, J = 5.49 Hz, 1H, 5′-OH), 5.32 (dm, $J_{2',F}$ = 54.93 Hz, 1H, H-2′), 6.02 (dd, $J_{1',F}$ = 15.87 Hz, 3.66 Hz, 1H, H-1′), 7.62 (s, 1H, H-6), 11.43 (s, 1H, NH); ES-MS (+ve mode) = 259.1 $(M+H)^+$; Anal. Calcd for $C_{11}H_{15}FN_2O_4$: C 51.16, H 5.85, N 10.85. Found: C 51.33, H 5.94, N 10.72.

4.12. 1-(β-D-Arabinofuranosyl)-5-hydroxylmethyluracil (18)

4.12.1. Method A

A mixture of 1-β-D-arabinofuranosyluracil **17** (0.25 g, 1.1 mmol), paraformaldehyde (0.06 g) and 0.5 N KOH (1.25 mL) was heated at 65 °C for 52 h. The reaction mixture was cooled to room temperature and neutralized with Dowex 50 W-X-8–H+resin. After stirring for 1 h, the resin was filtered off, and washed with water (50 mL). The combined filtrate and washings were evaporated in vacuo at room temperature to an oily residue. The residue was purified on a silica gel column using EtOAc/MeOH (92:8, v/v) as the eluent to yield **18** (0.025 g, 9%) as a solid; mp 162–164 °C; ^{1}H NMR (DMSO-d_6): δ 3.52–3.70 (m, 2H, H-5′), 3.73 (dd, J = 8.79 Hz, 4.80 Hz, 1H, H-4′), 3.90 (m, 1H, H-3′), 3.99 (m, 1H, H-$_{2'}$), 4.08–4.15 (m, 2H, 5–CH_2), 4.95 (t, J = 5.39 Hz, 1H, 5–CH_2O*H*), 5.01 (t, J = 5.11 Hz, 1H, 5′-OH), 5.45 (d, J = 4.35 Hz, 1H, 3′-OH), 5.53 (d, J = 5.11 Hz, 1H, 2′-OH), 6.00 (d, J = 4.34 Hz, 1H, H-1′), 7.58 (s, 1H, H-6), 11.31 (s, 1H, NH); ES-MS (+ve mode) = 297.1 $(M+Na)^+$; ES-MS (−ve mode) = 273.1 $(M-H)^-$; Anal. Calcd for $C_{10}H_{14}N_2O_7$: C 43.80, H 5.15, N 10.22. Found: C 43.94, H 5.36, N 10.05.

4.12.2. Method B

A mixture of **17** (0.25 g, 1.1 mmol), 37% formaldehyde (1.25 mL) and 0.5 N KOH (1.25 mL) was heated at 65 °C for 18 h. The reaction mixture was cooled to room temperature and neutralized with Dowex 50 W-X-8–H^+ resin. After stirring for 1 h, the resin was filtered off, and washed with water (50 mL). The combined filtrate and washings were evaporated in vacuo at room temperature to an oily residue. The residue was purified on a silica gel column using EtOAc/MeOH (92:8, v/v) as the eluent to yield **18** (0.086 g, 30%) as a solid. Physical data were identical to the product obtained by method A.

4.12.3. Method C

A mixture of **17** (0.25 g, 1.1 mmol), paraformaldehyde (0.06 g) and 0.5 N HCl (0.8 mL) was refluxed for 12 h. TLC showed decomposition of the starting material.

4.13. 1-(2,3,5-Tri-*O*-acetyl-β-D-arabinofuranosyl)-5-acetoxymethyluracil (19)

To an ice cooled (0 °C) solution of **18** (0.19 g, 0.42 mmol) in anhydrous pyridine (10 mL) was added acetic anhydride (0.21 g, 2.1 mmol) dropwise. The reaction mixture was stirred at 0 °C for

1 h and then at room temperature for 24 h. Pyridine was removed in vacuo followed by co-evaporation with ethanol (2 × 25 mL). The resulting residue was purified on a silica gel column using EtOAc/Hexane (70:30 v/v) as the eluent to yield **19** (0.13 g, 42%) as syrup; ^{1}H NMR ($CDCl_3$): δ 2.05, 2.07, 2.16 and 2.17 (4s, 12H, 4 × CH_3), 4.23 and 4.46 (2 m, 3H, H-4′, H-5′), 4.89 (s, 2H, 5-CH_2), 5.12 (m, 1H, H-3′), 5.41 (dd, J = 3.66 Hz, 1.22 Hz, 1H, H-2′), 6.30 (d, J = 4.27 Hz, 1H, H-1′), 7.73 (s, 1H, H-6), 8.46 (br s, 1H, NH); ES-MS (+ve mode) = 443.1 $(M+H)^+$, 465.0 $(M+Na)^+$; ES-MS (−ve mode) = 441.1 $(M-H)^-$.

4.14. 1-(2,3,5-Tri-*O*-acetyl-β-D-arabinofuranosyl)-4-thio-5-acetoxymethyluracil (20)

To a dried mixture of **19** (0.12 g, 0.27 mmol) and Lawesson's reagent (0.20 g, 0.48 mmol) was added anhydrous 1,4–dioxane (10 mL). The reaction mixture was refluxed for 3.5 h, cooled to rt and concentrated in vacuo. The crude product thus obtained was purified on a silica gel column using $CHCl_3$/MeOH (97.5:2.5 v/v) as the eluent to yield **20** (0.05 g, 40%) as a syrup. The product was deblocked as stated in the next step.

4.15. 1-(β-D-Arabinofuranosyl)-4-thio-5-hydroxymethyluracil (21)

Deprotection of **20** using the procedure as described for **6a**→**6** yielded compound **21**. Yield 1%; syrup; ^{1}H NMR (CD_3OD): δ 3.77–3.84 (m, 2H, H-5′), 3.94 (m, 1H, H-4′), 4.08 (m, 1H, H-3′), 4.17 (m, 1H, H-2′), 4.48 (s, 2H, 5–CH_2), 6.13 (d, J = 4.27 Hz, 1H, H-1′), 7.80 (s, 1H, H-6). Anal. Calcd for $C_{10}H_{14}N_2O_6S$: C 41.37, H 4.86, N 9.65. Found: C 41.11, H 4.80, N 9.86.

4.16. 1-(2,3,5-Tri-*O*-acetyl-β-D-arabinofuranosyl)-5-acetoxymethylcytosine (22)

To a solution of **19** (0.20 g, 0.45 mmol), triethylamine (0.09 g, 0.89 mmol) and 1-methylpiperidine (0.05 g, 0.50 mmol) in dry acetonitrile (10 mL) was added *p*-toluenesulfonyl chloride (0.17 g, 0.89 mmol) in three portions at 0 °C with stirring. The reaction mixture was stirred at 0 °C for 4 h. To this was added NH_4OH (5 mL) at rt and the reaction mixture stirred at rt for 15 min. Acetonitrile was removed in vacuo and the resulting residue was purified on a silica gel column using EtOAc/MeOH (94:6 v/v) as the eluent to yield **22** (0.06 g, 30%) as a syrup; ^{1}H NMR ($CDCl_3$): δ 2.09 and 2.14 (2s, 6H, 2 × CH_3), 2.17 (s, 6H, 2 × CH_3), 4.23–4.35 and 4.58–4.68 (2 m, 3H, H-4′, H-5′), 5.05 (m, 1H, H-3′), 5.09 (s, 2H, 5-CH_2), 5.42 (dd, J = 3.66 Hz, 1.22 Hz, 1H, H-$_{2'}$), 6.30 (d, J = 3.66 Hz, 1H, H-1′), 8.07 (s, 1H, H-6), 8.49 and 10.49 (2s, 2H, 2 × NH); ES-MS (+ve mode) = 442.1 $(M+H)^+$.

4.17. 1-(β-D-Arabinofuranosyl)-5-methoxymethylcytosine (24)

Treatment of **22** with K_2CO_3 using the procedure as described for **6a**→**6** provided a crude product, which was purified on HPLC to yield pure **24** (0.02 g, 54%) as a syrup; [HPLC *t*R 7.0 min, column, OmniSpher 5 C-18 (100 mm × 4.6 mm) (from Varian Inc.), mobile phase, CH_3OH-H_2O (40:60), flow rate, 1.0 mL/min, UV wavelength, 254 nm, column temperature, 25 °C]; $[\alpha]_D$ +83.41 (*c* 0.14, MeOH); ^{1}H NMR (DMSO-d_6): δ 3.20 (s, 3H, OCH_3), 3.53–3.62 (m, 2H, H-5′), 3.67–3.76 (m, 1H, H-4′), 3.85–3.89 and 3.90–3.96 (2 m, 2H, H-2′, H-3′), 4.09 (dd, J = 18.3 Hz, 12.2 Hz, 2H, 5-CH_2), 5.42 (br s, 4H, 4 × OH), 6.01 (d, J = 4.27 Hz, 1H, H-1′), 6.58 (br s, 1H, NH), 7.29 (br s, 1H, NH), 7.60 (s, 1H, H-6); ES-MS (+ve mode) = 288.0 $(M+H)^+$, 310.0 $(M+Na)^+$; ES-MS (−ve mode) = 285.9 $(M-H)^-$; Anal. Calcd for $C_{11}H_{17}N_3O_6$: C 45.99, H 5.96, N 14.63. Found: C 46.23, H 5.84, N 14.56.

4.18. In vitro antimycobacterial activity assay (*M. bovis*, *Mtb*, and *M. avium*)

M. bovis (BCG), *Mtb* (H37Ra), and *M. avium* (ATCC 25291) were obtained from the American Type Culture Collection, Rockville, MD. Antimycobacterial activity was determined using the microplate alamar blue assay (MABA).[26] Test compounds were dissolved in DMSO at 10 mg/mL and subsequent dilutions were made in 7H9GC medium (Difco Laboratories, Detroit, Michigan) in 96 well plates. For these experiments, each compound was tested at 100, 50, 25, 10, 5, 1, and 0.5 μg/mL in triplicate. The experiments were repeated two times and the mean percent inhibition is reported in Table 1. The standard deviations were within 10% of the mean value. Frozen mycobacterial inocula were diluted in 7H9GC medium and added to each well at a final concentration of 2.5 × 10^5 CFU/mL. Sixteen control wells consisted of eight with bacteria alone (B) and eight with medium alone (M). Plates were incubated for six days and then 20 μL of 10× alamar blue and 12.5 μL of 20% Tween 80 were added to one M and one B well. Wells were observed for an additional 24–48 h for visual color change from blue to pink and read by spectrophotometer (at excitation 530/525 nm and emission 590/535 nm) to determine OD values. If the B well became pink by 24 h (indicating growth), reagent was added to the entire plate. If the B well remained blue, additional M and B wells were tested daily until bacterial growth could be visualized by color change. After the addition of the reagent to the plate, cultures were incubated for 24 h and plates were observed visually for color change and read by spectrophotometer. MIC was defined visually as the lowest concentration of a compound that prevented a color change from blue to pink. Percent inhibition was calculated as (test well-M bkg./B well-M bkg.) × 100. The lowest drug concentration affecting an inhibition of ~50% was considered as the MIC_{50}. Similar methodology was used for all (three) mycobacteria strains. Cycloserine, rifampicin and clarithromycin were used as positive controls. As negative controls, DMSO (2, 1, 0.2, 0.02 μL), was added to the B well at concentrations similar to those of compound wells; M wells served as negative controls. In most of the experiments, the M wells gave an OD of 3000–4000, and the B wells had OD values ranging between 60,000–100,000.

4.19. Antimycobacterial activity against a drug-resistant strain of *Mtb*

The activity of compound **21** was determined against rifampicin-resistant *Mtb* H37Rv (ATCC 35838, resistant to rifampicin at 2 μg/mL) using a radiometric-BACTEC assay.[29] This assay detects the metabolism of ^{14}C-labelled palmitic acid, where evolving $^{14}CO_2$ is captured and counted as a measure of mycobacterial growth and metabolism. The growing inoculum (2.5–5.0 × 10^5 CFU/vial) was diluted in a BACTEC vial containing radiometric 7H12 (BACTEC 12B) medium and incubated at 37 °C. Two-fold dilutions of test compounds were delivered to the inoculum-containing BACTEC vials. Negative control vials consisted of medium with bacteria inoculum, medium with bacteria inoculum at 1:100, and medium alone. As reference drugs, rifampicin and isoniazid were used at their MIC_{90} concentration. All the vials were incubated at 37 °C, and the growth index (GI) was determined in a BACTEC 460 instrument until the GI of the 1:100 inoculum controls reached 30. Vials were read daily and a change in GI (δ GI) was recorded for each compound. Percent inhibition was defined as (GI of test sample/GI of control) × 100. For the no drug control, the δ GI continued to increase and was much higher than the 1:100 inoculum control. The BACTEC assay was preferred with the resistant strain, because the method provides a safe, enclosed and biocontained method to monitor the kinetics of drug inhibition.

4.20. In vitro cytotoxicity assay

Human hepatoma cell line (Huh-7) was used to determine the effect of test compounds on human cell cytotoxicity using XTT and ^{3}H-Thymidine assays. Cell viability was measured using the cell proliferation kit II (XTT; Roche), as per manufacturer's instructions. Briefly, a 96 well plate was seeded with Huh-7 cells at a density of 2×10^4 cells per well. Cells were allowed to attach for 6–8 h when the medium was replaced with medium containing compounds at concentrations of 200, 100, 50, 10 and 1 μg/mL. DMSO was also included as control. Plates were incubated for two days at 37 °C. The color reaction involved adding 50 μL XTT reagents per well and incubating for 4 h at 37 °C. Plates were read on an ELISA plate reader (Abs 450–500 nm). For the ^{3}H-Tdr incorporation assay, Huh-7 cells were plated at 1×10^4 cells/well in 96 well flat bottom plates. Medium containing compounds at concentrations of 200, 100, 50, 10 and 1 μg/mL was added to the plate in triplicates. DMSO was also included as control. Plates were incubated for two days at 37 °C. The wells were pulsed with 0.5 μCi/well [^{3}H]-thymidine (Amersham) for 12–18 h. After this the plates were harvested on filter papers (Perkin Elmer) using a 96 well plate harvester (Tomtech MACH III M). The levels of [^{3}H]-thymidine incorporated into the DNA of proliferating cells were counted in a Microbeta Trilux liquid scintillation counter (Perkin–Elmer).

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